

## Effects of Adenine Arabinoside on Cellular Immune Mechanisms in Humans

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In vitro lymphocyte blastogenic responses to the commonly employed mitogens, phytohemagglutinin, pokeweed, and concanavalin A, were evaluated when adenine arabinoside (ara-A) in a concentration of 3  $\mu$ g/ml was added to the culture materials. Similarly, blastogenic and cytotoxic responses to cell cultures persistently infected with herpes simplex virus 1, herpes simplex virus 2, and varicella-zoster virus were determined in the presence of ara-A. No depression of these cellular immune responses by ara-A was demonstrated. This was in contrast to the effect of cytosine arabinoside, which at a concentration of 3  $\mu$ g/ml severely inhibited these immune responses. Further studies examined lymphocyte blastogenic responses to the mitogens and blastogenic and cytotoxic responses specific for the herpes group virus infecting patients who were subsequently treated with ara-A; determinations were made before, during, and after treatment. In vitro responses during and after treatment with ara-A were unchanged or often enhanced as compared to pretreatment values. Therefore, the antiviral chemotherapeutic agent, ara-A, does not appear to depress the host's cellular immune responses, which are vital to successful elimination of invading herpes group viruses.

Advances in the control of viral disease have depended on the development of vaccines and more recently on antiviral chemotherapy (2). The efficacy of antiviral chemotherapeutic agents has been primarily based on clinical response and the ability of an agent to eradicate the virus. However, full recovery from the viral disease still appears to require intact host defense mechanisms. Such host defense against viral infection involves a complex interplay between antibody and cell-mediated responses, and, although the relative importance of cellular immunity has not been fully delineated, the severe or fatal infections in children with thymic deficiency syndromes or patients on immunosuppressive therapy suggest that cellular immunity may be the major host factor in combating viral disease (9). Therefore, antiviral chemotherapeutic agents that depress the host cellular immune response might actually prevent adequate elimination of the virus.

The present studies were undertaken to evaluate the effect of adenine arabinoside (ara-A) on mechanisms of cellular immunity. Preliminary studies examined lymphocyte blastogenic responses to mitogens and blastogenic and cyto-

toxic responses to cell cultures persistently infected with herpes simplex virus types 1, and 2 (HSV-1 and -2) or varicella-zoster (V-Z) virus, with or without ara-A added to the culture material. Subsequent studies examined lymphocyte blastogenic and cytotoxic responses of patients before, during, or after treatment with ara-A.

### MATERIALS AND METHODS

**Preparation of lymphocytes.** Lymphocytes were separated from peripheral whole blood by centrifugation on a Hypaque-Ficoll gradient as previously described (12). The cell suspension was washed twice with culture medium, and the lymphocytes were adjusted to a concentration of  $2 \times 10^6$ /ml for determinations of blastogenesis and  $5 \times 10^6$ /ml for cytotoxicity assays in RPMI 1640 medium containing 20% autologous serum.

**Mitogen stimulation.** Using a biopipette, 0.1 ml of the lymphocyte suspension ( $2 \times 10^6$  lymphocytes) was added to an equal volume of various concentrations of the mitogens, phytohemagglutinin (PHA), pokeweed (PWM), and concanavalin A (Con A), in one well of sterile flat-bottom microtest plates (3040 Micro Test II tissue culture plates, Falcon Plastics, Division of Becton, Dickinson and Company, Oxnard, Calif.). All

cultures were prepared in triplicate, and five dilutions of PHA and PWM and four dilutions of Con A were included along with control cultures containing lymphocytes incubated with medium alone. The plates were covered with sterile plastic lids and cultures were incubated for 72 h. Five hours prior to harvest, 0.05 ml of RPMI 1640 medium containing 1  $\mu$ Ci of [ $^3$ H]thymidine was added to each well. A harvesting apparatus previously described (8) and technically refined for our use (Biomedical Research Institute, Rockville, Md.) was employed for separation of the stimulated lymphocytes on glass-fiber filters, for washing of these cells and for recovery of radioactive uptake. The glass fiber disks were then dried in an oven and transferred to vials containing 5 ml of scintillation fluid (TLA in toluene, Beckman Instruments, Inc., Fullerton, Calif.) for counting in a Beckman liquid scintillation spectrometer. The average counts per minute of triplicate samples were determined, and results were expressed as a blastogenic index (BI): counts of [ $^3$ H]thymidine uptake per min for lymphocytes incubated with mitogen divided by uptake after incubation with medium alone.

**Cell cultures persistently infected with herpes group viruses.** Cell line MA-160 derived from human prostate adenoma tissue was inoculated with the VR<sub>1</sub> strain of HSV-1, the MS strain of HSV-2, or with V-Z virus at a multiplicity of 1:1. The development of the resulting three persistently infected cell lines is described in a separate communication. A control cell line of MA-160 and the infected lines were cultured with Eagle minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, Eagle non-essential amino acids, 1 mM sodium pyruvate, 100 U of penicillin per ml, and 100  $\mu$ g of streptomycin per ml. For these studies, aliquots of the three infected cell lines and the control line in suspensions of  $2 \times 10^6$  cells/ml were control rate frozen with 7.5% dimethylsulfoxide as the cryoprotective agent and stored in the vapor phase of a liquid nitrogen refrigerator.

Both the control and virus-infected cell lines were monitored prior to use for surface membrane antigen by indirect immunofluorescence. An average of 60 to 70% of the infected cells fluoresced; control cells did not fluoresce.

Transfer of frozen cells from Bethesda, Maryland to Fort Sam Houston, Texas was accomplished in nitrogen vapor transport refrigerators via air freight. On arrival at the immunology laboratories of Brooke Army Medical Center, the ampoules were rapidly thawed in a 37 C water bath, diluted in culture medium, and washed three times in medium by centrifugation at  $150 \times g$ . The cells were then resuspended in RPMI 1640 medium and subsequently processed for the blastogenic and cytotoxic assays as described below.

**Assays of HSV-1, HSV-2, and V-Z-induced lymphocyte blastogenesis.** Methods were similar to those employed for a one-way mixed lymphocyte culture (11). Stimulating cells, however, included one of the infected cultures and uninfected MA-160 cells. These stimulating cells were concentrated to  $2 \times 10^6$  cells/ml and incubated for 1 h with 20  $\mu$ g of mitomy-

cin C per ml. They were then washed three times and resuspended in RPMI 1640 medium with *N*-2-hydroxyethyl-piperazine-*N'*-2'-ethanesulfonic acid buffer. Lymphocytes were purified as described above and adjusted to a final concentration of  $2 \times 10^6$ /ml in RPMI 1640 containing 20% autologous serum. The lymphocyte suspension ( $2 \times 10^6$  lymphocytes) in 0.1 ml was mixed with an equal volume of stimulating cells in one well of microtest plates, as described for mitogen stimulation.

Preliminary experiments were performed to determine the optimal incubation time and the optimal concentration of stimulating cells for these assays of lymphocyte blastogenesis. Maximum response was observed after 5 to 6 days of incubation and with a lymphocyte-to-stimulating cell ratio of 1:1. Therefore, for the present assays  $2 \times 10^6$  lymphocytes were combined with  $2 \times 10^6$  infected or control cells and incubated for 5 days.

All cultures were prepared in triplicate. Twenty-four hours prior to harvest, 0.05 ml of RPMI 1640 medium containing 1  $\mu$ Ci of [ $^3$ H]thymidine was added to each well. Harvesting and counting of radioactive uptake was accomplished as described above for mitogen stimulation.

**Assay of lymphocyte cytotoxicity.** A  $^{51}$ Chromium ( $^{51}$ Cr) microassay of lymphocytotoxicity to the cell lines persistently infected with HSV-1, HSV-2, or V-Z was used in these studies. Details of the microassay were described previously (8). Briefly, this technique examines lymphocyte-target cell interaction, employing the infected cell lines described above as the target cells. The quantitative release of  $^{51}$ Cr from the target cells is used as an index of lymphocyte-mediated cytotoxicity against the infected cells. Uninfected MA-160 cultures served as control target cells to quantitate  $^{51}$ Cr release not attributable to the virus itself. Target cell suspensions were labeled with  $^{51}$ Cr (sodium chromate- $^{51}$ Cr, New England Nuclear; specific activity, 50 to 500 Ci/g) by incubating  $10^7$  cells in 1 ml of media containing 100  $\mu$ Ci of  $^{51}$ Cr for 45 min as described previously (8).

An aliquot of 0.1 ml of lymphocytes ( $5 \times 10^6$ ) was incubated with 0.1 ml of target cells ( $5 \times 10^6$  cells) in flat-bottom microtest plates. Target cells were also combined with 0.1 ml of media alone to determine spontaneous release of  $^{51}$ Cr. All cultures on the microtest plate were prepared in triplicate. The plates were covered with sterile plastic lids and placed on a rocker platform at 37 C in a 5% CO<sub>2</sub> atmosphere. The cell suspensions were harvested after 24 h of incubation with the apparatus described above. The total amount of  $^{51}$ Cr available for release was determined by placing an aliquot of 0.1 ml of the target cell suspension directly in a gamma-counting tube, and zero time samples were also harvested at the time incubation of the assay materials was begun. To determine lymphocyte-mediated cytotoxicity specific for HSV-1, spontaneous release of  $^{51}$ Cr from infected target cells (*I*) and the control cells (*C*) was subtracted from that released from the interaction of lymphocytes with infected target cells (*I'*) or control cells (*C'*), respectively. The release at zero time was

subtracted from the total amount of  $^{51}\text{Cr}$  in infected cells ( $T_i$ ) and control ( $T_c$ ) target cells to determine the actual amount that was available for release during the incubation period. Finally, the specific immune release (SIR) was calculated by subtracting the percentage of  $^{51}\text{Cr}$  release in the control target cells from that released from the infected ones, according to the following formula:  $\% \text{SIR} = [I^L - I/T_i - \text{zero time}_i - C^L - C/T_c - \text{zero time}_c] \times 100$ .

**Study cases.** To date 11 patients who have been treated with ara-A have been studied with the assays described. These assays included blastogenic responses to mitogens and blastogenic and cytotoxic responses specific to the virus causing the patients' infection. Data from four representative patients are included in this report. These patients were an adult with HSV-1 encephalitis, a neonate with generalized HSV-2 infection, an adult with Hodgkin's disease and a child with leukemia, both infected with V-Z virus. Two of these four patients expired from the infection during treatment; these deaths were not related to administration of the drug.

**Antiviral agents.** ara-A was obtained from Parke, Davis & Co., along with the phosphate-buffered saline used as the placebo for their double blind treatment protocol. Both the drug and the placebo were examined in vitro in the initial experiments. Cytosine arabinoside (ara-C) (Cytosar, The Upjohn Co., Kalamazoo, Mich.) was also included in the in vitro studies. These agents were diluted with RPMI so that a final concentration of  $3 \mu\text{g/ml}$  was achieved for ara-A, the phosphate-buffered placebo, or ara-C, and the effect of these products on mechanisms of cellular immunity was examined.

## RESULTS

**Mitogen stimulation.** In the preliminary experiments, the influence of ara-A on lymphocyte blastogenic responses to mitogens was examined (Fig. 1, 2, and 3). The experiments were repeated five times with five different lymphocyte donors, and results were averaged

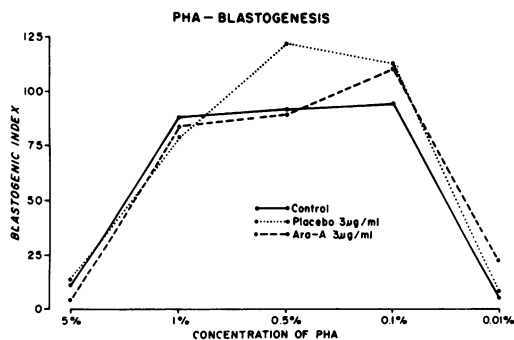


FIG. 1. *In vitro* lymphocyte blastogenesis after incubation with PHA expressed as a blastogenic index: counts per minute of  $[^3\text{H}]$ thymidine uptake for lymphocytes incubated with PHA divided by uptake after incubation with media alone.

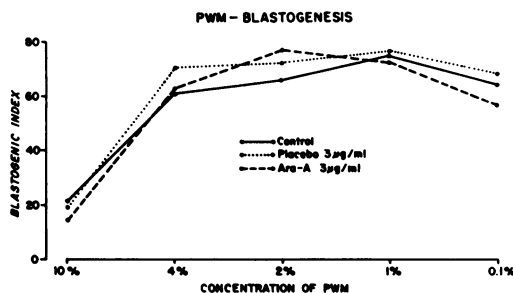


FIG. 2. *In vitro* lymphocyte blastogenesis after incubation with PWM expressed as a blastogenic index: counts per minute of  $[^3\text{H}]$ thymidine uptake for lymphocytes incubated with PWM divided by uptake after incubation with media alone.

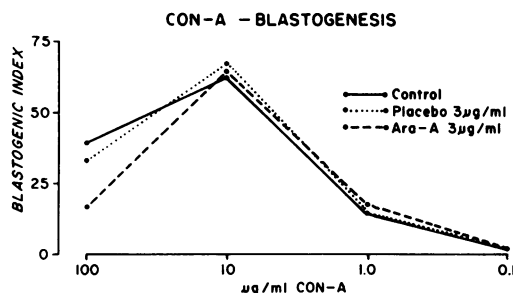


FIG. 3. *In vitro* lymphocyte blastogenesis after incubation with Con A expressed as a blastogenic index: counts per minute of  $[^3\text{H}]$ thymidine uptake for lymphocytes incubated with Con A divided by uptake after incubation with media alone.

for these three figures. The assays were conducted using a 72-h incubation with a 5-h  $[^3\text{H}]$ thymidine pulse. No significant differences were observed for these assays when either  $3 \mu\text{g}$  of ara-A per ml was added to tissue culture materials or an equivalent concentration of placebo.

These assays of mitogen-induced lymphocyte blastogenesis were also conducted with variable incubation periods ranging from 1 to 7 days and, likewise, no difference in responses was demonstrated when ara-A in a concentration of  $3 \mu\text{g/ml}$  was added to the culture medium.

**Blastogenic and cytotoxic responses to herpes group viruses.** Tables 1 and 2 summarize the results of lymphocyte blastogenesis and lymphocyte cytotoxicity to herpes group-infected target cells in the presence of ara-A. Persistently infected cell lines included cells infected with HSV-1, HSV-2, and V-Z virus. No significant difference was noted for the assays which included ara-A in the tissue culture medium.

**ara-C.** In contrast to ara-A, ara-C severely

depressed blastogenic responses to mitogens and blastogenic responses to the cell lines persistently infected with HSV-1, HSV-2, or V-Z. The maximum BI after stimulation with PHA, PWM, or Con A in the presence of ara-C remained less than 3 for repeated determina-

tions. The maximum BI after incubation with the herpes group viruses was less than 2. The cytotoxic response to herpes group viruses was also decreased to approximately one-half the control value, but this appeared secondary to toxic effects of ara-C on the cell lines themselves.

No patients were treated with ara-C, so studies comparable to those undertaken for patients on ara-A could not be obtained.

**Patients treated with ara-A.** Data from four representative patients treated with ara-A and studied with the assays described are presented (Table 3). There were no statistically significant differences in results obtained before, during, or after therapy with ara-A. For these and other patients studied by our laboratory, parameters of cellular immunity have usually increased during treatment with this antiviral chemotherapeutic agent. No significant depression of cellular immune responses has yet been observed.

## DISCUSSION

Historically, the most severe limiting factor in the use of antiviral chemotherapeutic agents has been their relatively high frequency of severe toxic side effects. Both idoxuridine and ara-C are known to produce bone marrow toxicity (2, 4, 6). Leukopenia predisposes patients to life-threatening bacterial, mycotic, and protozoan diseases, and thrombocytopenia results in

TABLE 1. *Lymphocyte Blastogenesis for herpes group viruses in the presence of ara-A*

Media contents	Infected cell culture		
	HSV-1	HSV-2	V-Z
Ara-A	12.1 <sup>a</sup>	18.1	12.0
Placebo	12.3	15.8	11.5
Control	10.7	17.1	9.6

<sup>a</sup> Expressed as a blastogenic index: counts of [<sup>3</sup>H]thymidine uptake per minute for lymphocytes incubated with infected cells divided by uptake after incubation with uninfected control cells.

TABLE 2. *Lymphocyte cytotoxicity to herpes group-infected target cells in the presence of ara-A*

Media contents	Infected cell cultures		
	HSV-1	HSV-2	V-Z
Ara-A	27.8 <sup>a</sup>	15.7	10.6
Placebo	24.9	10.0	6.1
Control	28.3	12.5	7.2

<sup>a</sup> Expressed as percent specific immune release of <sup>51</sup>Cr attributable to intracellular virus.

TABLE 3. *Cellular immune reactions of patients treated with ara-A*

Patient	Age	Agent	Underlying disease	Mitogens <sup>a</sup>			Blastogenesis <sup>a,b</sup>	Cytotoxicity <sup>b</sup> (%)
				PHA	PWM	Con A		
1	38 years	HSV-1	None	31 <sup>c</sup>	72	67	9.2	12.1
				49 <sup>d</sup>	57	43	5.3	24.7
				47 <sup>e</sup>	60	60	12.3	31.4
2	4 days	HSV-2	None	162	73	90	0.9	8.9
				187	65	81	1.2	13.5
3	8 years	V-Z	Leukemia	87	43	73	7.8	23
				99	57	65	15.3	26
				68	51	54	13.1	17
4	23 years	V-Z	Hodgkin's	18	12	9	0.7	3
				8	2	5	0.7	7

<sup>a</sup> Expressed as blastogenic index: counts of [<sup>3</sup>H]thymidine uptake per minute for lymphocytes incubated with mitogens or infected cells divided by uptake after incubation with media or uninfected control cells, respectively.

<sup>b</sup> Responses specific for the virus with which the patient was infected.

<sup>c</sup> Before treatment.

<sup>d</sup> During treatment.

<sup>e</sup> After treatment.

serious risk of uncontrolled hemorrhage. Immune suppression of the humoral immune system predisposes patients to risk from secondary infections (1, 3, 10). Gastrointestinal and central nervous system toxicity also limit the usefulness of some of the potential antiviral compounds. Ara-C and idoxuridine have both been shown to inhibit cerebellar development in fetal and young animals, causing serious concern in recommending their use in neonatal deoxyribonucleic acid viral disease (5). Minor but irritating side effects such as oral mucosal ulceration and alopecia are unfortunately common.

Experience to date with parenteral administration of ara-A has been strikingly devoid of serious toxic side effects. This has been the experience even though, in contrast to ara-C and idoxuridine, ara-A has a much longer half-life of antiviral activity, principally afforded by the activity of its primary hypoxanthine derivative metabolite (P. E. Borondy, D. R. Mourer, T. Chang, and A. J. Glazko, *Fed. Proc.* **32**:777, 1973).

Since full recovery from severe viral infections appears to require at least partial host immune competence, it is imperative that antiviral chemotherapeutic agents not suppress these host responses. Previous reports have demonstrated that therapeutic doses of ara-A do not produce immunosuppression in animals (7; Z. S. Zam, Y. M. Centifanto, and H. E. Kaufman, *Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother.*, 14th, San Francisco, Calif., Abstr. 139, 1974), and further investigation has revealed that antibody production is not impaired after therapy with ara-A (Zam et al., *Progr. Abstr. Intersci. Conf. Antimicrob. Agents Chemother.*, 14th, San Francisco, Calif., Abstr. 139, 1974).

The present study has evaluated assays of cellular immunity when ara-A is added to in vitro materials and has examined mechanisms of cellular immunity in patients treated with ara-A for various deoxyribonucleic acid virus infection. With the assays employed there is no evidence that the antiviral agent, ara-A, impairs these host defense mechanisms.

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